

1 Effects of riparian plant diversity loss on aquatic microbial decomposers become more  
2 pronounced at longer times

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1 Abstract

2 We examined the potential long-term impacts of riparian plant diversity loss on  
3 diversity and activity of aquatic microbial decomposers. Microbial assemblages were  
4 obtained in a mixed-forest stream by immersion of mesh bags containing three leaf  
5 species (alder, oak and eucalyptus), commonly found in riparian corridors of Iberian  
6 streams. Simulation of species loss was done in microcosms by including a set of all  
7 leaf species, retrieved from the stream, and non-colonized leaves of three, two or one  
8 leaf species. Leaves were renewed every month throughout 6 months, and microbial  
9 inoculum was ensured by a set of colonized leaves from the previous month. Microbial  
10 diversity, leaf mass loss and fungal biomass were assessed at the 2nd and 6th month  
11 after plant species loss. Molecular diversity of fungi and bacteria, as the total number of  
12 operational taxonomic units per leaf diversity treatment, decreased with leaf diversity  
13 loss. Fungal biomass tended to decrease linearly with leaf species loss on oak and  
14 eucalyptus, suggesting more pronounced effects of leaf diversity on lower quality  
15 leaves. Decomposition of alder and eucalyptus leaves was affected by leaf species  
16 identity, mainly after long time of diversity loss. Leaf decomposition of alder decreased  
17 when mixed with eucalyptus, while decomposition of eucalyptus decreased in mixtures  
18 with oak. Results suggest that effects of leaf diversity on microbial decomposers  
19 depended on leaf species number and also on which species were lost from the system,  
20 especially after longer times. This may have implications for the management of  
21 riparian forests to maintain stream ecosystem functioning.

22

23 Keywords: leaf decomposition, litter diversity, litter quality, microbial diversity,  
24 streams

## 1 Introduction

2 Human activities are affecting freshwater ecosystems worldwide leading to irreversible  
3 changes in biotic communities and the processes they support [1, 2]. A key ecological  
4 process in freshwaters is plant-litter decomposition, which is driven by microorganisms  
5 and invertebrate detritivores [3, 4]. Both aquatic fungi and bacteria play a key role in  
6 organic-matter decomposition by converting plant litter into a more nutritious food  
7 source for invertebrate detritivores [5]. Fungi have been recognized as the major  
8 microbial decomposers in streams accounting for more than 90% to the total biomass  
9 production on decomposing leaves [6]. However, the role of bacteria cannot be  
10 neglected because its contribution to plant-litter decomposition increases along time as  
11 smaller-size detritus are being produced [7, 8]. Protozoa (e.g., ciliates) can exert a top-  
12 down predation pressure on aquatic bacterial communities [9]; however, its role in  
13 plant-litter decomposition remains practically unexplored (but see [10, 11]).

14 Due to the importance of plant-litter decomposition and its tractability in field and  
15 microcosm experiments, the scientific community is increasingly using this process to  
16 better understand the relationships between biodiversity and ecosystem functioning in  
17 freshwaters [12, 13]. In fact, several studies have provided evidence of how leaf-litter  
18 decomposition is shaped by the diversity of resources [14] and of consumers (fungi [15-  
19 17]; invertebrates [18-20]).

20 Several studies indicate that composition and diversity of riparian plant species  
21 influence the diversity of aquatic fungi [14, 21]. The quality of plant-litter mixtures can  
22 also influence microbial biomass; for instance, the presence of high quality leaves of  
23 *Liriodendron tulipifera* in litter mixtures led to an increase of fungal and bacterial  
24 biomasses, while the presence of low quality leaves of *Rhododendron maximum* led to  
25 an opposite effect [22]. Most studies have focused on composite samples in litter

1 mixtures (but see [23]), but examining the effects of litter diversity loss within  
2 individual litter species might help to better understand the contribution of individual  
3 litter species for overall diversity effects on litter decomposition.

4 Microbes have faster growth rates than other organisms involved in plant-litter  
5 decomposition in streams, such as invertebrate detritivores. Generally, maximum  
6 doubling times of fungi on leaf litter decomposing in streams range from 5 to 50 days  
7 [24]. Therefore, microbes can have several generations throughout plant-litter  
8 decomposition, and may show strong responses to alterations in litter diversity at  
9 relatively short-time scales.

10 The aim of this study was to examine how aquatic microbial decomposers respond to  
11 riparian plant diversity loss. We used a microcosm approach to monitor the  
12 development of leaf-associated microbial assemblages during 6 months after inducing  
13 the loss of plant species from the system. Microbial assemblages were obtained in a  
14 mixed-forest stream by immersion of a pool of three leaf species (alder, oak and  
15 eucalyptus) commonly found in the riparian corridors of Iberian streams. After 2 and 6  
16 months, we assessed leaf mass loss, fungal biomass, and diversity of fungi, bacteria and  
17 ciliates associated with individual leaf species. We expected that plant-litter mixtures,  
18 containing litter species with different chemical composition, would provide better  
19 resources to support higher microbial diversity and/or activity. We also expected that  
20 any constrain to microbial biomass development or leaf decomposition of lower-quality  
21 leaf species could be overcome by the presence of high quality leaf species in mixtures.

22

23 Methods

24 Microbial colonization of leaves in a stream

1 In October 2009, leaves from alder (*Alnus glutinosa* (L.) Gaertn.), oak (*Quercus robur*  
2 L.) and eucalyptus (*Eucalyptus globulus* Labill.) were collected from trees immediately  
3 before abscission and dried at room temperature. Leaves were soaked in deionised  
4 water, cut into disks, and sets of 12 leaf disks (four disks per plant species) were placed  
5 in fine-mesh bags (0.5-mm diameter pore size). On 28 October 2009, 28 bags  
6 containing leaf species mixtures were immersed in a mixed-forested stream in Portugal,  
7 the Estorãos stream (8.63800°W, 41.78194°N), to allow microbial colonization.  
8 At the study site, the stream was about 0.5 m deep and 2.5 m wide, the stream bed was  
9 constituted by rocks, pebbles and gravel, and the riparian vegetation was dominated by  
10 *A. glutinosa*, *Q. robur* and *E. globulus*. During leaf colonization, stream water had on  
11 average ( $\pm$ SEM) a temperature of  $14 \pm 1.0$  °C, a pH of  $5.9 \pm 0.06$ , a conductivity of  $31$   
12  $\mu\text{S cm}^{-1}$  and a redox potential of  $51 \pm 1.5$  mV (Multiline F/set 3 no. 400327; WTW,  
13 Weilheim, Germany). Nutrient concentrations in the stream water were  $0.30 \pm 0.04$  mg  
14  $\text{L}^{-1}$  of  $\text{N-NO}_3^-$ ,  $0.003 \pm 0.000$  mg  $\text{L}^{-1}$  of  $\text{N-NO}_2^-$ ,  $<0.01$  mg  $\text{L}^{-1}$  of  $\text{N-NH}_3$  and  $<0.003$  P-  
15  $\text{PO}_4^{3-}$  (HACH kit, programs 351, 371, 385, and 490, respectively; HACH, Loveland,  
16 CO, USA).

17

#### 18 Microcosm setup

19 After two weeks of stream immersion, mesh bags containing mixtures of alder, oak and  
20 eucalyptus leaves were brought to the laboratory. Simulation of leaf species loss was  
21 done in microcosms (500 mL Erlenmeyer flask with sterile stream water) containing  
22 non-colonized disks of three leaf species (30 disks per species, one treatment), two leaf  
23 species (45 disks per species, three treatments) or one leaf species (90 disks, three  
24 treatments) enclosed in mesh bags, and 12 leaf disks (four per species) retrieved from  
25 the stream as microbial inoculum. After 30 days, 12 leaf disks of each microcosm with

1 treatments of three, two or one leaf species were kept as inoculum, and the remaining  
2 leaf disks were replaced by non-colonized leaf disks keeping leaf species treatments.  
3 This procedure was repeated every 30 days during 6 months. Four replicates were used  
4 per treatment. Microcosms were kept, aseptically, under aeration, with artificial light at  
5 16 °C, and stream water was renewed every 15 days. After 2 and 6 months, leaf disks  
6 were used to assess leaf mass loss, fungal biomass, and fungal, bacterial and ciliate  
7 diversity by denaturing gradient gel electrophoresis (DGGE), after PCR amplification of  
8 microbial DNA with specific primers targeting each microbial group.

9

#### 10 Leaf mass loss

11 Leaf disks from all microcosms of each individual leaf species were freeze-dried for  
12 two days and weighed to the nearest 0.01 mg. Mass loss of each leaf species was  
13 expressed as percentage of the respective initial dry mass.

14

#### 15 Fungal biomass

16 Fungal biomass was estimated from ergosterol concentration associated with  
17 decomposing leaf disks according to Gessner [25]. Briefly, lipids from each individual  
18 leaf species were extracted from sets of 6 leaf disks by heating (80 °C for 30 minutes) in  
19 8 g L<sup>-1</sup> KOH-methanol. The lipid extract was purified by solid-phase extraction (Sep-  
20 Pak cartridges, Waters, Milford, MA) and ergosterol was quantified by high  
21 performance liquid chromatography (Beckmann Gold System, Brea, CA, USA), at 282  
22 nm, using a LiChrospher RP18 column (250×4 mm, Merck). The system was run  
23 isocratically with methanol as mobile phase (1.4 mL min<sup>-1</sup>, 33 °C).

24

#### 25 Microbial diversity

1 DNA was extracted from 4 freeze-dried disks of each leaf species with a soil DNA  
2 extraction kit (MoBio Laboratories, Solana Beach, California), according to the  
3 manufacturer instructions. The ITS2 region of fungal ribosomal DNA (rDNA) was  
4 amplified with the primer pair ITS3GC and ITS4 [26]; V3 region of 16S bacterial  
5 rDNA was amplified with the primer pair 338GC and 518 [26]; and 18S rDNA of  
6 ciliates was amplified with the primer pair 984GC and 1147 (adapted from [27]). For  
7 PCR of fungal, bacterial and ciliate DNA, 2x of GoTaq® Green Master Mix (Promega),  
8 0.4  $\mu\text{M}$  of the appropriate primers and 1 to 10  $\mu\text{L}$  of DNA ( $1\text{-}10\text{ ng } \mu\text{L}^{-1}$ ) were used in a  
9 final volume of 25  $\mu\text{L}$ .

10 PCRs were carried out in a MyCycler Thermal Cycler (BioRad Laboratories, Hercules,  
11 CA, USA). The PCR program for bacteria and fungi was: initial denaturation at 95 °C  
12 for 2 min; 36 cycles of denaturation at 95 °C for 30 s; primer annealing at 55 °C for 30 s  
13 and extension at 72 °C for 1 min; and final extension at 72 °C for 5 min [26]. The PCR  
14 program for ciliates was: initial denaturation at 94 °C for 5 min; 30 cycles of  
15 denaturation at 94 °C for 45 s; primer annealing at 55 °C for 60 s and extension at 72 °C  
16 for 90 s; and final extension at 72 °C for 7 min [27].

17 DGGE analysis was performed using a DCode™ Universal Mutation Detection System  
18 (BioRad Laboratories, Hercules, CA, USA). For fungi, 700 ng of the amplified DNA  
19 products with 380-400 bp were loaded on 8% (w/v) polyacrylamide gel in 1x Tris-  
20 acetate-EDTA (TAE 1x) with a denaturing gradient from 30 to 70% (100% denaturant  
21 corresponds to 40% formamide and 7 M urea). For bacteria, 700 ng of the amplified  
22 DNA products with ca. 200 bp were loaded on 8% (w/v) polyacrylamide gel in 1x TAE  
23 with a denaturing gradient from 40 to 75%. For ciliates, 700 ng of the amplified DNA  
24 products with 750-800 bp were loaded on 6% (w/v) polyacrilamide gel in 1x TAE with  
25 a denaturing gradient from 30 to 42.5%. Fungal and bacterial DNA was separated at 55

1 V and 56 °C, while ciliate DNA was separated at 80 V and 60 °C. All gels were run for  
2 16 h. Gels were stained with 1x of GelStar (Lonza) for 10 min, and gel images captured  
3 under UV light in a gel documentation system (GenoSmart; VWR).

4

5 Nutrient content in leaves

6 To estimate initial carbon and nitrogen in leaves, samples of alder, oak and eucalyptus  
7 leaves were grounded with a ball mill and ca.100 mg of powdered leaf material was  
8 analyzed in a LECO-CNS 2000, using EDTA as a standard. Analyses were done in  
9 C.A.C.T.I. – Centro de Apoio Científico e Tecnológico á Investigación – University of  
10 Vigo, Spain.

11 Initial quality of leaves as C:N ratio differed between the three leaf types as follows:  
12 alder ( $13.29 \pm 0.26$ ) < oak ( $19.69 \pm 0.71$ ) < eucalyptus ( $30.51 \pm 0.26$ ).

13

14 Statistical analyses

15 DGGE gels of microbial DNA were aligned and normalized using Gelcompar II  
16 (Applied Maths, Sint-Martens-Latem, Belgium), and each DGGE band was considered  
17 an operational taxonomic unit (OTU).

18 Linear regressions were used to establish the relationships between leaf species number  
19 and total number of OTUs of each microbial group per leaf treatment. The distribution  
20 of fungi, bacteria and ciliates associated with leaf species identity and number at each  
21 time (2 and 6 months) was analysed by Correspondence Analysis (CA, [28])  
22 downweighting the contribution of rare species. Data of fungal, bacterial and ciliates  
23 communities' structure were based on OTUs, as the relative intensity of each band in  
24 DGGE fingerprinting. Data was square root transformed.



1 For each leaf type, a three-way nested ANOVA was used to test if leaf species number,  
2 leaf species identity (nested within species number) and time after diversity loss  
3 significantly affected leaf mass loss and fungal biomass [29]. In punctual cases, in  
4 which diversity effects were marginally significant, a two-way nested ANOVA was  
5 done, testing for the effects of leaf species number and identity (nested with leaf species  
6 number) at each time separately. Because the experimental design was unbalanced, we  
7 applied Type III analyses of variance using the Variance Estimation and Precision  
8 (VEPAC) module in Statistica 8.0 (Statsoft, Tulsa, OK, USA). Differences between  
9 treatments were analysed using the Tukey-Kramer's post-test, which is a modification  
10 of the Tukey's post-test for unbalanced number of samples [29]. Linear regressions  
11 were used to establish the relationships between leaf species number and fungal  
12 biomass for each leaf species.

13 Linear regressions were done in Prism 4.0 for Windows (GraphPad software Inc., San  
14 Diego, CA), analyses of variance were done in Statistica 8.0 for Windows (Statsoft,  
15 Inc., Tulsa, OK) and multivariate analyses were done in CANOCO 4.5 for Windows  
16 (Microcomputer Power, Ithaca, NY).

17

## 18 Results

### 19 Effects of plant litter diversity on microbial diversity

20 Molecular diversity of microbial communities on decomposing leaves showed a total of  
21 41, 64 and 29 operational taxonomic units (OTUs) for fungi, bacteria and ciliates,  
22 respectively (Fig. 1). In a general way, communities of fungi, bacteria and ciliates on  
23 each leaf species differed between single- and mixed-species treatments (Fig. 1 and Fig.  
24 2). The number of fungal OTUs on individual leaf species was higher in mixed-species  
25 treatments than in single species treatments, particularly at the longer time, i.e. after 6

1 months of leaf species loss (Fig. 2). Conversely, bacterial diversity on individual leaf  
2 species was generally higher in single-leaf species treatments than in mixed-species  
3 treatments (e.g. 46 OTUs on oak alone versus 35-38 OTUs on mixtures, after long time  
4 of leaf diversity loss). The diversity of ciliates appeared to decrease with time because  
5 lower number of OTUs was found after long time in microcosms (except for oak  
6 leaves). Similarly to that found for bacterial diversity, the number of ciliate OTUs on  
7 individual leaf species was higher in single leaf species treatments than in mixed species  
8 treatments (Fig. 2).

9 However, when taking into account the total number of OTUs associated with all leaf  
10 species composing a given mixture, positive relationships were found between leaf  
11 species number and fungal or bacterial diversity (linear regression,  $P=0.0003$  and  
12  $P=0.024$ , respectively; Fig. 3). For ciliates, that relationship was not significant (linear  
13 regression,  $P=0.065$ ). The dependence of microbial diversity on leaf species number  
14 strongly increased from ciliates to bacteria to fungi (slopes were 2.0, 4.2 and 6.0  
15 OTUs/unit of leaf species diversity, respectively).

16 CA ordination of fungal assemblages according to leaf species number, leaf species  
17 identity and time after leaf diversity loss showed that the 1<sup>st</sup> factor explained 20.8% of  
18 the total variance in fungal assemblages, and separated assemblages established at short  
19 time from those established at long time (Fig. 4a). The 2<sup>nd</sup> factor explained 15.2% of the  
20 total variance and distinguished assemblages according to leaf species identity and leaf  
21 species number, mainly separating fungal assemblages on 3 leaf species from the others.

22 CA ordination of bacterial assemblages showed that the first two factors, explaining  
23 24.5% of the total variance, separated assemblages on oak leaves from the others, and  
24 further discriminated assemblages according to leaf species number and the time after  
25 diversity loss (Fig. 4b). The 1<sup>st</sup> CA factor of ciliate assemblages explained 19.1% of the

1 total variance and separated assemblages by the time after diversity loss, while the 2<sup>nd</sup>  
2 factor explaining 15.1% of the total variance mainly distinguished assemblages  
3 according to leaf species number (Fig. 4c).

4

#### 5 Effects of plant litter diversity on leaf decomposition

6 Leaf mass loss varied between 26% in microcosms containing eucalyptus mixed with  
7 oak after long time of leaf diversity loss and 43% in microcosms with oak in mixtures  
8 with alder and eucalyptus at short time (Fig. 5). Decomposition of alder leaves was not  
9 affected by leaf species number or time after leaf species loss, but effects of leaf identity  
10 were marginally significant (3-way nested ANOVA, Table 1). However, the effects of  
11 leaf identity on decomposition of alder leaves became stronger after long time of leaf  
12 diversity loss (2-way nested ANOVA,  $P=0.005$ ,  $F=11.59$ ). Decomposition of alder  
13 leaves was higher in mixtures with oak than in mixtures with eucalyptus or mixtures  
14 with oak and eucalyptus (Tukey-Kramer's tests,  $P=0.024$  and  $P=0.048$ , respectively).  
15 Decomposition of oak leaves was affected by leaf species number and interaction  
16 between species number and time after leaf species loss (3-way nested ANOVA, Table  
17 1); leaf mass loss was higher in mixtures of 3 species than in treatments with oak alone  
18 (Tukey-Kramer's test,  $P=0.040$ ). Mass loss of eucalyptus leaves was affected by time  
19 after leaf diversity loss and marginally affected by leaf identity (3-way nested ANOVA,  
20 Table 1). Similarly to that found for alder leaves, the effects of leaf species identity on  
21 decomposition of eucalyptus leaves became stronger after long time of leaf diversity  
22 loss (2-way nested ANOVA,  $P=0.011$ ,  $F=9.12$ ). Mass loss of eucalyptus leaves was  
23 higher when eucalyptus was mixed with alder leaves than with oak leaves (Tukey-  
24 Kramer's test,  $P=0.046$ ).

25

1 Effects of plant litter diversity on fungal biomass  
2 Leaf-associated fungal biomass varied between 55  $\mu\text{g}$  ergosterol  $\text{g}^{-1}$  leaf dry mass, in  
3 alder leaves in mixtures with eucalyptus after long time of leaf diversity loss, and 468  
4  $\mu\text{g}$  ergosterol  $\text{g}^{-1}$  leaf dry mass in oak leaves in mixtures with alder at the shorter time  
5 (Fig. 5). Fungal biomass on alder leaves was affected by leaf species number (3-way  
6 nested ANOVA, Table 1), with higher values in mixtures with three leaf species than  
7 with two leaf species (Tukey-Kramer's test,  $P=0.024$ ). Fungal biomass on oak leaves  
8 was not affected by leaf species number, leaf identity or time after leaf diversity loss (3-  
9 way nested ANOVA, Table 1). However, when effects of leaf diversity were analysed at  
10 the longer time after diversity loss, leaf species number affected fungal biomass on oak  
11 leaves (2-way nested ANOVA,  $P=0.017$ ,  $F=7.04$ ). Moreover, fungal biomass on oak  
12 leaves decreased linearly with leaf species loss after long time (Linear regression,  
13  $F=16.34$ ,  $P=0.002$ ,  $r^2=0.62$ , not shown). Fungal biomass associated with eucalyptus  
14 leaves was affected by leaf species number and time after leaf diversity loss (3-way  
15 nested ANOVA, Table 1), with overall higher biomass at the longer time (Tukey-  
16 Kramer's test,  $P=0.006$ ). The loss of leaf species led to a linear decrease in fungal  
17 biomass on eucalyptus leaves, with a stronger relationship at longer time (Linear  
18 regression,  $F=5.89$ ,  $P=0.036$ ,  $r^2=0.37$ , at short time;  $F=15.70$ ,  $P=0.003$ ,  $r^2=0.61$ , at long  
19 time; not shown).

20

## 21 Discussion

22 Our study suggests that changes in plant species diversity of riparian corridors affect  
23 diversity and activity of microbes on decomposing plant litter in streams. The leaf  
24 species (alder, oak and eucalyptus) used in our study provide resources with different  
25 quality due to differences in their leaf C:N ratio. Thus, each leaf species might harbour

1 different microbial assemblages that could provide inoculum to the different leaf species  
2 constituting the mixtures. Indeed, molecular diversity (as number of OTUs) of fungi on  
3 individual leaf species tended to be higher in leaf species mixtures. However, this trend  
4 was not observed on bacterial and ciliate diversity, which was higher in single species  
5 treatments. Fungi are reported to have antagonistic interactions with bacteria during leaf  
6 decomposition [30], but fungi have morphological and physiological adaptations that  
7 allow them to colonize plant litter earlier than bacteria [31], which might be  
8 outcompeted by fungi [32]. In addition, a reduction of bacterial diversity by the  
9 presence of fungi may decrease ciliate diversity because ciliates feed on bacteria and  
10 show preference for certain bacterial species [9]. This is consistent with the positive  
11 linear relationship between bacterial and ciliate diversity found in our study (not  
12 shown).

13 Although microbial assemblages on individual leaf species have shown different  
14 responses to leaf diversity loss, when microbial diversity as overall number of OTUs  
15 per leaf species treatment was considered, a positive relationship was found between  
16 leaf species diversity and the diversity of fungi and bacteria. A positive co-variation of  
17 fungal diversity with riparian plant species diversity was previously documented [14,  
18 21]. Also, the replacement of native mixed forests by monocultures of eucalyptus in  
19 riparian corridors of streams in the Iberian Peninsula decreased the diversity of aquatic  
20 fungi with shifts in community composition [33]. In our study, the decrease in the  
21 number of OTUs per unit of leaf species lost was particularly high for fungi (6  
22 OTUs/unit of leaf species diversity), pointing to a higher dependence of fungi than  
23 bacteria or ciliates on the diversity of plant litter resources. This agrees with the major  
24 role of fungi in early stages of plant-litter decomposition in streams [6, 7]; fungi have an  
25 efficient enzymatic machinery to degrade polysaccharides of plant cell walls, and their

1 hyphae have high ability to penetrate substrates [31]. The relationship between fungal  
2 diversity and litter diversity found in our study (6 OTUs decrease per unit of leaf  
3 species lost) was even stronger than reported by others (1.7 fungal species decrease per  
4 unit of leaf species lost [21]). This apparent discrepancy might be related to differences  
5 in the levels of leaf litter diversity investigated (1-3 leaf species in our study versus 7-17  
6 leaf species in Laitung and Chauvet [21]). The positive relationship between the  
7 diversity of resources (litter) and the diversity of consumers (fungi) can be explained by  
8 mechanisms of niche differentiation [34]. We expected that more leaf species would  
9 provide a greater variety of resources that could allow the co-existence of more fungal  
10 species. However, it is conceivable that above a certain leaf diversity level, further  
11 increases in leaf diversity will not provide a proportional increase in nutrient supply or  
12 habitat structures for fungi. Therefore, the dependence of fungal diversity on plant litter  
13 diversity is expected to be stronger at lower leaf diversity levels.

14 The shifts in the structure of microbial assemblages on decomposing leaves in response  
15 to plant species loss were accompanied by changes in decomposition of oak leaves, but  
16 not of alder or eucalyptus leaves. However, the identity of litter mixture affected leaf  
17 decomposition of alder and eucalyptus leaves, mainly after long time of leaf diversity  
18 loss. Actually, the composition of litter mixtures appears to have a greater role in leaf  
19 decomposition in streams than diversity of litter species [35-37] with the differences in  
20 litter quality explaining the effects of leaf identity on leaf decomposition [38-40]. In our  
21 study, decomposition of eucalyptus leaves (higher C:N ratio, lower quality) tended to be  
22 faster when mixed with alder (lower C:N ratio, higher quality) than with oak leaves  
23 (intermediate C:N ratio), suggesting that microbial assemblages on eucalyptus leaves  
24 might have benefited from the presence of compounds released by high quality leaves to  
25 fulfil their metabolic needs [13]. Conversely, we found a deceleration of decomposition

1 of alder leaves by the presence of eucalyptus at the longest time after leaf species loss.  
2 Also, fungal biomass on alder leaves was consistently lower when mixed with  
3 eucalyptus, especially at the longest time. Eucalyptus leaves contain oils and tannic  
4 acids that inhibit the growth of aquatic fungi [41]. Thus, if inhibitory compounds were  
5 leached from eucalyptus leaves to the surrounding environment [13], microbial activity  
6 on other leaf species composing the mixture might also be inhibited.

7 In our study, the effects of leaf diversity were stronger on fungal biomass and diversity  
8 than on microbially-driven leaf decomposition. Moreover, fungal biomass and diversity  
9 tended to decrease as litter species were lost from the system, especially for oak and  
10 eucalyptus. This may have implications for freshwater invertebrates that preferentially  
11 feed on leaves colonized by microbes [42, 43]. Moreover, fungal diversity correlates  
12 positively with leaf consumption rates by invertebrate shredders [14]. Thus, the effects  
13 of leaf diversity loss on fungal diversity and biomass observed in our study might have  
14 indirect impacts on plant-litter decomposition in streams.

15 Overall, leaf litter diversity and quality changed the structure of microbial assemblages  
16 and affected leaf decomposition and fungal biomass on individual litter species. Fungal  
17 biomass tended to decrease with leaf species loss, especially for lower quality leaf  
18 species (oak and eucalyptus) after long time of diversity loss. Leaf decomposition was  
19 mainly affected by leaf species identity at the longer time. Microorganisms growing on  
20 low quality leaves appeared to benefit from the presence of other leaf species, as shown  
21 by higher fungal biomasses found in leaf mixtures. Conversely, the presence of  
22 eucalyptus lowered the decomposition of alder leaves at the longer time after leaf  
23 diversity loss. The eucalyptus species used in our study was introduced in the Iberian  
24 Peninsula almost two centuries ago, and nowadays vast areas are covered by  
25 monocultures of this exotic tree [44]. Alterations in diversity and quality of riparian

1 vegetation can jeopardize litter inputs into streams with possible bottom-up effects to  
2 the functioning of detritus food-webs [45]. Thus, protecting and/or restoring riparian  
3 vegetation is important to conserve microbial diversity and maintain the functioning of  
4 detritus food-webs in freshwaters.

5

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25

1 Figure captions

2

3 **Fig. 1** DGGE patterns of DNA of fungal, bacterial and ciliate assemblages on individual  
4 leaf species (A, *Alnus glutinosa*; O, *Quercus robur*; E, *Eucalyptus globulus*) from  
5 single- and mixed-leaf species treatments, after short (2 months) and long time (6  
6 months) of leaf diversity loss. M, marker used to align different gels belonging to the  
7 same microbial group

8

9 **Fig. 2** Number of OTUs from DGGE analyses of fungal, bacterial and ciliate  
10 assemblages associated with individual leaf species (A, *Alnus glutinosa*; O, *Quercus*  
11 *robur*; E, *Eucalyptus globulus*) from single- and mixed-leaf species treatments, after  
12 short (2 months) and long time (6 months) of leaf diversity loss

13

14 **Fig. 3** Relationship between the number of OTUs of fungi (a), bacteria (b) and ciliates  
15 (c) and leaf species diversity. In mixtures of two and three leaf species, data represent  
16 total number of OTUs per leaf species treatment. Data were fitted to linear regressions.  
17 Fungi,  $Y=5.98X+7.25$ ,  $r^2=0.69$ ,  $P=0.0003$ ; Bacteria,  $Y=4.21X+39.50$ ,  $r^2=0.36$ ,  $P=0.024$ ;  
18 Ciliates,  $Y=1.99X+6.25$ ,  $r^2=0.28$ ,  $P=0.065$

19

20 **Fig. 4** CA diagrams for ordination of fungal (a), bacterial (b) and ciliate (c) OTUs  
21 according to exposure time (short and long), leaf species number (1sp, 2sp and 3sp) and  
22 identity (alder, oak and eucalyptus)

23

1 **Fig. 5** Percentage of leaf mass loss and fungal biomass from individual leaf species  
2 alone and in mixtures, after short (2 months) and long time (6 months) of leaf diversity  
3 loss. Values are mean + SEM; n=3 for fungal biomass and n=4 for leaf mass loss  
4

1 Table 1. Effects of leaf species number (Sp n°), leaf species identity (ID), nested within  
 2 leaf species number, and time after leaf diversity loss (T) on leaf mass loss and fungal  
 3 biomass. In leaf mixtures, data came from individual leaf species

Parameter	Leaf species	Factor	SS	DF	F	P
Leaf mass loss	Alder	Sp n°	26.7	2	0.47	0.630
		T	75.8	1	2.67	0.115
		Sp n°*T	20.5	2	0.36	0.701
		ID (Sp n°)	117.2	1	4.13	0.053
		ID (Sp n°)*T	52.1	1	1.84	0.188
		Error	680.7	24		
	Oak	Sp n°	115.0	2	3.77	0.038
		T	58.1	1	3.81	0.063
		Sp n°*T	122.7	2	4.02	0.031
		ID (Sp n°)	8.8	1	0.58	0.455
		ID (Sp n°)*T	14.0	1	0.91	0.349
		Error	366.4	24		
	Eucalyptus	Sp n°	26.4	2	0.63	0.539
		T	132.9	1	6.40	0.018
		Sp n°*T	90.1	2	2.17	0.136
		ID (Sp n°)	87.4	1	4.21	0.051
		ID (Sp n°)*T	33.2	1	1.60	0.218
		Error	498.9	24		
Fungal biomass	Alder	Sp n°	39497.4	2	6.75	0.007
		T	221.1	1	0.08	0.787
		Sp n°*T	11955.3	2	2.04	0.162
		ID (Sp n°)	4051.0	1	1.38	0.257
		ID (Sp n°)*T	3468.7	1	1.19	0.292
		Error	46814.2	16		
	Oak	Sp n°	73834.0	2	3.05	0.076
		T	94.0	1	0.01	0.931
		Sp n°*T	6609.0	2	0.27	0.765
		ID (Sp n°)	18124.0	1	1.50	0.239
		ID (Sp n°)*T	11212.0	1	0.93	0.350
		Error	193740.0	16		
	Eucalyptus	Sp n°	27038.0	2	10.90	0.001
		T	10665.5	1	8.60	0.010
		Sp n°*T	2211.0	2	0.89	0.430
		ID (Sp n°)	1000.1	1	0.81	0.383
		ID (Sp n°)*T	34.0	1	0.03	0.871
		Error	19851.3	16		

4